ORIGINAL ARTICLE

Astrovirus Gastroenteritis in Children in Taipei

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Background/Purpose: A prospective study was initiated to study astroviral infections in Taiwan.
Methods: A total of 415 stool samples were collected and assayed for astrovirus antigen using an enzyme immunoassay.
Results: Twelve (2.9%) stool samples from 12 patients were positive for astrovirus antigen. Most patients (8/12) had watery diarrhea which lasted for 2–6 days. The majority of patients recovered without specific treatment, except for two patients who were treated with antibiotics for possible bacterial infections. One patient developed chronic diarrhea and two episodes were nosocomially acquired. A clustering in the autumn and winter, with a peak in December (5/12), was noted. Growth on Caco-2 cells was performed for four specimens with positive astroviral RT-PCR results, and a characteristic cytopathic effect was observed after 4 days. Astroviral RNA was detected in six stool samples using RT-PCR. Five of six strains were serotype 1 and one strain was serotype 3. Sequence homology among the six strains was between 80.5% and 100%. A higher degree of homology (89.9–100%) was noted in the five strains of serotype 1. A phylogenetic study demonstrated two clusters in our strains and Oxford reference strain types 1 and 2.
Conclusion: Our study results provide further information about the prevalence and span of clinical spectra associated with astroviral infections in Taiwan. The current study showed that infection with astroviruses may be an important cause of gastroenteritis, as well as respiratory symptoms, in infants and children in Taipei. [*J Formos Med Assoc* 2008;107(4):295–303]

Key Words: astrovirus, gastroenteritis, pediatrics, prevalence, Taiwan

Astroviruses belong to the family *Astroviridae*, and were first detected by electron microscopy in 1975 in the stools of infants with mild diarrhea and vomiting.[1,2] The non-enveloped virion is small, with a diameter of 28 nm, and contains a single-stranded positive-sense RNA of 6.8 kb. The viral nucleic acid consists of open reading frames (ORF), including ORF1a, ORF1b and ORF2.[3] Electron microscopy results have demonstrated a characteristic five- or six-pointed star morphology.[4,5] In addition to humans, astroviruses have also been observed in the feces of calves, cats, ducklings, lambs and pigs.[6] Astroviruses are hardy and relatively resistant to extreme changes in pH and heat, ultraviolet irradiation, and the levels of chlorine typically found in swimming pools.[7]

Serologic surveys in the UK and USA have shown that >70% of children acquire astrovirus infection by 5 years of age.[8] Epidemiologic studies have indicated that astroviruses are a significant cause of gastroenteritis in children, with a prevalence ranging from 2.7% to 8.6%.[9–13] This

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prevalence rate is second only to that of rotaviruses in Thailand.[10] In addition, astroviruses are known to have induced outbreaks in hospitals, day-care settings, kindergartens, junior high schools, and in military recruits.[14–18]

Most of the astroviral illnesses occur during the winter months in temperate climates and predominantly affect children < 7 years of age.[19] After an incubation period of 1–3 days, infants infected with astroviruses present with mild diarrhea without fever or vomiting.[8] As a result of difficulties in viral culture, the diagnosis has relied on electron microscopy or immunoelectron microscopy, but these methods of diagnosis have low sensitivity.[5,20,21] The detection rate has increased, however, since new astroviral assays have become available. The more sensitive assays include an enzyme immunoassay (EIA), RNA probe hybridization, and RT-PCR.[11,22–25] The advent of an EIA kit to detect astrovirus antigen in stool samples has greatly improved the diagnosis of astroviral infections. This is evident in view of the large capacity of EIA to handle multiple samples in a short period of time. Furthermore, in contrast to immunoelectron microscopy, the sensitivity of EIA is 91% and the specificity is 96%.[22] The development of a typing immunoassay (TYPE-EIA) makes the serotyping of astroviruses and performing epidemiologic studies both easy and feasible.[2,26,27]

Rotaviruses and enteric adenoviruses are the two most well-recognized causes of viral gastroenteritis in children. However, information concerning other enteric pathogens is inadequate. The current study was conducted to better understand the prevalence of astrovirus infections in Taiwanese children. In addition, the dominant serotypes of astroviruses were determined and a phylogenetic study was done.

Methods

Stool specimens were prospectively collected from children with gastroenteritis attending the outpatient and emergency departments, and pediatric wards of the National Taiwan University Hospital between 1 July 1998 and 30 June 1999. Samples were stored at −70°C until the time of assay.

Definitions

An episode of diarrhea was defined as the passage of unformed stools with at least twice the usual daily frequency in 24 hours. Nosocomial gastroenteritis was defined as the onset of diarrhea and/or vomiting beginning at least 72 hours after admission or < 72 hours after discharge.

Assay of stool astrovirus antigen

All specimens were assayed using an IDEIA™ Astrovirus Test (DAKO Diagnostics Ltd., Ely, Cambridgeshire, UK). The test utilized a combination of genus-specific monoclonal and polyclonal antibodies to detect known strains of human astroviruses. At a photometric determination of 450 nm, the negative control should be < 0.150 absorbance units and the positive control must have a value > 0.5 absorbance units. Specimens were considered to be positive when the absorbance value was greater than the cut-off value, which was calculated by adding 0.150 absorbance units to the negative control value.

Extraction of RNA and RT-PCR

Astrovirus RNA was extracted from stool specimens with a positive EIA result using a commercial kit (RNaid kit; Bio 101 Inc., Cambridgeshire, UK) with a modification of a published procedure.[28] Briefly, stool samples were suspended in 50 mM Tris-HCl (pH 7.5) in an approximately 10% suspension (v/v) and were clarified by centrifugation at 1000 rpm for 15 minutes at 4°C. Then, 10 μL RNaid matrix was added to a mixture of 200 μL stool supernatant and 200 μL 6 M guanidine thiocyanate, and the sample was vortexed and mixed on a rocker (Vortex Genie-2, Bohemia, NY, USA) at room temperature for 10 minutes. Each sample was centrifuged for 1 minute at 10,000 rpm at room temperature, and the supernatant was removed by aspiration. The pellet was then washed three times with 500 μL RNA wash solution and centrifuged at 10,000 rpm for 1 minute. After removing the

supernatant, the pellet was dried under vacuum for 10 minutes. The RNA was eluted with 45 μL preheated DEPC-treated water, mixed well, and incubated at 65°C for 10 minutes. The suspension was then centrifuged at 10,000 rpm for 2 minutes and the supernatants were maintained in sterile Eppendorf tubes and stored at −20°C until needed for RT-PCR.

RT-PCR

The primers used for RT-PCR, MON 340 and MON 348, were chosen according to a previous report.[14] MON 340 (5′ ACA CAT TAT TTG TTG TCA TAC T 3′) and MON 348 (5′ ACA TAT GCT GCT GTT ACT ATG 3′) are located within ORF1a and yield an amplicon of 289 bp that corresponds to positions 1182–1470 on the HastV-2 genome.[11] Five microliters of RNA and 1 μL (6 pmol/μL) of each primer (MON 340 and MON 348) were boiled at 95°C for 10 minutes and immediately chilled on ice for 5 minutes. The RNA solution was mixed with 43 μL of a mixture that contained 32 μL DEPC-treated ddH_2O, 10 μL 5× reaction mix, and 1 μL Fast-Run™ RT-PCR Kit (Protech, Taipei, Taiwan). The first strand cDNA was synthesized at 50°C for 30 minutes followed by 94°C for 2 minutes. The second strand and PCR reaction were performed by 30 cycles of amplification using denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, extension for 1 minute at 72°C, and final extension for 7 minutes at 72°C. All amplification reactions were performed in a model 9600 thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT, USA). PCR products were analyzed by electrophoresis in 2% agarose gels and visualized by ultraviolet illumination after staining with ethidium bromide. The PCR product was 289 bp.

Nucleotide sequencing

Forty microliters of RT-PCR product was purified using a commercial kit (PCR Clean-up Kit; Viogene, Taipei, Taiwan). Four microliters of purified DNA was amplified directly with the dideoxynucleotide sequencing kit of a dye terminator with primers (MON 340 and MON 348) and Tag FS, and then sequenced by an ABI sequencing machine (373A; Applied Biosystems, Foster City, CA, USA). Homologies were figured by DNASIS 7.0 software (Hitachi Software Engineering, San Francisco, CA, USA). The percentage homology was calculated based on how many matching nucleotides existed over the number of overlapped nucleotides. The sequences were further analyzed by using MEGA, version 1.02 (Center for Evolutionary Functional Genomics, Tempe, AZ, USA) for the Kimura-2 parameter distance and neighbor-joining to produce the phylogenetic tree.

Serotyping

RT-PCR for serotyping was conducted according to the procedure described in the previous section. Primers were chosen from a previous report.[29] End is the 3′ end of the capsid region: End (−), 5′-TCC TAC TCG GCG TGG CCG C-3′ (1–19 upstream from the 3′ end in the capsid region). AST-S1, AST-S2, AST-S3, AST-S4, AST-S5, AST-S6 and AST-S7 were designed from the sequences that were reported in the nucleotide sequence database: AST-S1 (+), 5′-AAC CAA GGA ATG ACA ATG AC-3′ (212–193 upstream from the 3′ end of End); AST-S2 (+), 5′-ACC TGC GCT GAG AAA CTG-3′ (158–141 upstream from the 3′ end of End); AST-S3 (+), 5′-CTG CTT GCA TCT GGT CTT TCA-3′ (119–99 upstream from the 3′ end of End); AST-S4 (+), 5′-TGA TGA TGA AGA AGA CTC TAA TAC-3′ (258–235 upstream from the 3′ end of End); AST-S5, 5′-TAG TAA CTT ATG ATA GCC-3′ (388–371 upstream from the 3′ end of End); AST-S6, 5′-TGG CCA CCC TTG TTC CTC AGA-3′ (427–407 upstream from the 3′ end of End); and AST-S7, 5′-CTA GAC AAC AAC ACC CCG-3′ (548–531 upstream from the 3′ end of End).

Culture in Caco-2 cells

Caco-2 cells (ATCC-HTB37) were maintained in minimal essential medium (MEM) with Earle's salts supplemented with non-essential amino acids (splMEM) and 10% fetal bovine serum (GIBCO, Grand Island, NY, USA). Ten percent stool suspensions were prepared in 0.5% PBS gelatin, vortexed for 1 minute, and clarified at 2000 rpm for

15 minutes at 4°C. Tubes (15 mL) of Caco-2 cells that were > 95% confluent were inoculated with 200 μL of the suspension, treated with chloroform, and incubated for 2 hours at 37°C without rocking. After adding 1 mL splMEM, the incubation was continued and the cytopathic effect was monitored.

Results

Clinical symptoms, gender, age distribution and seasonal patterns

The relevant data of 12 patients with astroviral infections, as defined by a positive EIA, were analyzed. The male-to-female ratio was 9:3 and the median age was 11 months (range, 1–91 months). Most episodes of diarrhea occurred in the fall and winter months (August to February), with a peak in December (42%). Five patients presented with watery diarrhea that lasted for 2–6 days. Bloody stools were observed in three patients. One had acute myeloid leukemia, neutropenia, fever and thrombocytopenia. Another patient, who had cerebral palsy, subsequently recovered. The third patient developed hematochezia for 3 days and experienced an uneventful course. Abdominal pain occurred in two patients, one of whom was put on antibiotic therapy because of suspected salmonellosis. Three-quarters of the patients were febrile and half had involvement of the respiratory tract, which manifested as cough and rhinorrhea. A skin rash was observed in one patient. There were two patients with nosocomial infections, Cases 8 and 12. Case 8 had underlying type Ia glycogen storage disease and developed diarrhea on day 21 in hospital. Case 12, a premature infant with a birth weight of 1520 g at 30 weeks' gestational age, acquired an astrovirus infection on day 40 in hospital, and, therefore, fulfilled our definition of a nosocomial infection. A 14-month-old boy (Case 1) suffered from chronic diarrhea for more than 1 month and the stool specimen was positive by EIA and RT-PCR (serotype 1). Diagnostic efforts to identify other intestinal pathogens were negative.

EIA, RT-PCR and nucleotide sequencing

A total of 415 specimens were tested for astrovirus by EIA between July 1, 1998 and June 30, 1999, and 12 specimens (2.9%) from 12 patients were positive. RT-PCR was not performed in three specimens due to an insufficient quantity of specimen. RNA from the nine positive specimens was subjected to RT-PCR with astrovirus-specific primers MON 340 and MON 348. Six samples yielded the expected product of 289 bp (Figure 1A). The nonstructural coding regions ORF1a, between MON 340 and MON 348, were sequenced. Simple homology in the region was 80.5–100% in nucleic acids. The intraserotypic homology of serotype 1 isolates in our series (strains 1 and 6, 4 and 5) reached 100% (Table 1). The phylogenetic tree showed two clusters in Oxford reference strains 1 and 2 and our strains. Strain 3 (serotype 3) was significantly different from the other strains and reference strain types 1 and 2 (Figure 2).

Serotyping and growth on Caco-2 cells

Serotyping was performed by RT-PCR. Extracted RNA was reverse transcribed and amplified with End and a serotype-specific primer. At first, End and S1-specific primer were used in six specimens with positive RT-PCR results from the previous procedure. Of six samples, five yielded an amplification product of 212 bp (serotype 1). The remaining specimen was transcribed and amplified with primers End and serotype-specific primers individually (AST-S2 to AST-S7), and the product was 119 bp (serotype 3). The culture supernatant of type 3, provided by Dr H. Ushijima, also yielded the same result (Figure 1B). Following the inoculation of Caco-2 cells with stool specimens, characteristic cytopathic effects were observed on the fourth day following inoculation with four specimens (Cases 1–4).

Other pathogens

Studies for rotavirus, adenoviruses type 40 and 41, *Salmonella* spp., *Shigella* spp., or *Campylobacter jejuni* were completed at the discretion of the attending physician. However, no pathogens other than astrovirus were identified in specimens

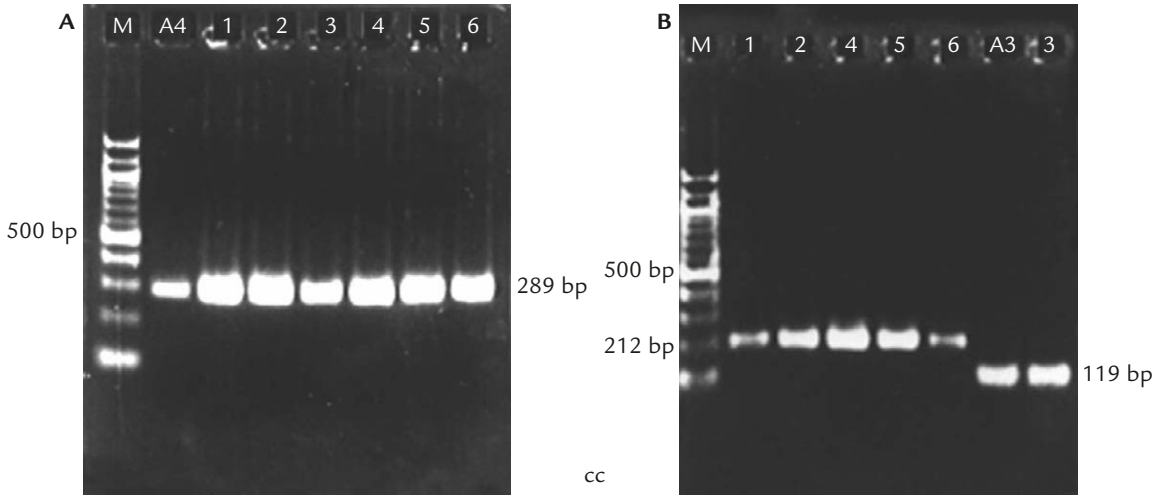

Figure 1. (A) Results of RT-PCR on samples with a positive EIA and standard serotypes (from Dr H. Ushijima) using the primers, MON 340 and MON 348. M = 100 bp DNA ladder; A4 = serotype 4; 1, 2, 3, 4, 5 and 6 indicate cases. (B) Results of RT-PCR on serotyping of strains 1 to 6 using primers End and serotype-specific primers. M = 100 bp DNA ladder; 1, 2, 4, 5 and 6 indicate cases; A3 = serotype 3 (from Dr H. Ushijima); 3 = Case 3.

Table 1. Percent homology in nucleotide sequences in ORF1a among reference strain serotypes 1 and 2, and virus strains 1–6 from patients in National Taiwan University Hospital*†

	MON 340 and MON 348							
	AST-S1	AST-S2	1 (S1)	2 (S1)	3 (S3)	4 (S1)	5 (S1)	6 (S1)
AST-S1	–							
AST-S2	91.9	–						
1 (S1)	89.8	92.7	–					
2 (S1)	89.8	91.9	99.6	–				
3 (S3)	80.5	80.5	83.7	83.7	–			
4 (S1)	98.4	91.9	91.7	91.1	81.3	–		
5 (S1)	98.4	93.1	91.1	89.4	81.3	100	–	
6 (S1)	89.8	93.1	100	99.6	83.3	91.1	91.1	–

**246 nucleotides inside the primer regions were compared; †AST-S1, AST-S2, Oxford reference strain serotypes 1 and 2, were obtained from GenBank (accession nos. Z25771 and L13745).*

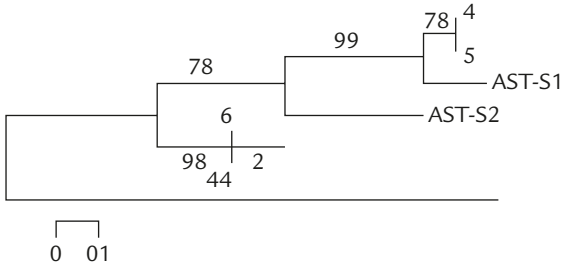

Figure 2. Phylogenetic tree demonstrating the genetic relatedness of astrovirus serotypes 1 and 2, and our strains 1–6. AST-S1 and AST-S2, sequences of Oxford reference strains, were obtained from GenBank (accession nos. Z25771 and L13745).

that showed the presence of astrovirus antigens (Table 2).

Discussion

In the current study, astrovirus antigen was detected in 2.9% of stool specimens (12 patients), which was comparable with the results of previous studies.[9–13] The reason for the low prevalence in our study may in part be due to the fact

Table 2. Characteristics of 12 patients with astrovirus infection

Case	Gender	Age (mo)	Month	Diarrhea per 24 hr (duration)	Vomiting	Fever	Other symptoms	Underlying disease	EIA	RT-PCR	Serotype	Culture
1	M	14	Oct	+, 3, (2)	–	+	Cough, rhinorrhea	–	0.676	+	Type 1	+
2	F	16	Dec	W, Mu, 4, (4)	+	+	Skin rash	–	0.752	+	Type 1	+
3	F	46	Dec	+	+	–	Cough	–	1.104	+	Type 3	+
4	M	8	Dec	+, 5	–	+	–	Chronic diarrhea	1.553	+	Type 1	+
5	M	2	Jan	W, 6, (5)	–	+	Cough	*E. coli* sepsis	0.517	+	Type 1	ND
6	M	3	Feb	W, 6, (3)	+	+	Cough	–	0.883	+	Type 1	ND
7	M	8	Aug	W, B	–	+	–	AML, neutropenic fever	0.254	–	ND	ND
8*	M	5	Sep	W, 4, (4)	–	–	–	GSD Ia	0.278	–	ND	ND
9	F	46	Aug	W, 6	+	+	Abdominal pain	Salmonellosis	0.276	–	ND	ND
10	M	91	Oct	W, B	+	+	Cough, hematochezia	GI bleeding	0.339	ND†	ND†	ND†
11	M	69	Dec	W, B, 4, (4)	+	–	Abdominal pain, cough, rhinorrhea	Cerebral palsy	0.259	ND†	ND†	ND†
12*	M	1	Dec	W, 4, (6)	–	–	–	–	0.335	ND†	ND†	ND†

**Nosocomial infection; †specimens were not sufficient to perform RT-PCR, serotyping, and culture in Caco-2 cells. EIA = enzyme immunoassay; RT-PCR = reverse transcription-polymerase chain reaction; M = male; F = female; W = watery; Mu = mucoid; B = bloody; AML = acute myeloid leukemia; GSD Ia = glycogen storage disease Ia; GI = gastrointestinal; ND = not done.*

that the majority of stool specimens studied were obtained from the wards and the emergency department, and these patients were prone to more severe symptoms than outpatients.[30] In addition, the quality of specimens and the timing of specimen acquisition may also have been contributing factors.

Many reports have indicated that RT-PCR is a sensitive method for the detection of astrovirus from cultures and feces.[18,31–34] Nevertheless, our detection rate was 67% (6/9) in comparison with EIA. The low detection rate may be due to a low viral load in specimens or the decay of viral RNA from repeated thawing and freezing.[35] The nucleotide sequencing of ORF1a exhibited 80.5–100% homology between Oxford reference strain serotypes 1 and 2 and our six strains. The intraserotypic homology of serotype 1 (reference strain and strains 1, 2, 4, 5 and 6) was 89.8–100%. The phylogenetic analysis showed two clusters according to our sequencing results of the reference strain types 1 and 2, and our strains. Strain 3 (type 3) was found to be different from the other strains, unlike in a previous study.[14] The homology was just 80.5%, in comparison to serotypes 1 and 2 (Oxford strains). Phylogenetic analysis of the nucleotide sequences of the PCR products derived from the reference strains and several wild isolates indicated two distinct genogroups of sequences in ORF1a. These genogroups correlated with the serotype. Genogroup A corresponded to types 1 to 5 and genogroup B corresponded to types 6 and 7.

We analyzed the clinical presentations of 12 patients with positive EIAs. The median age was 11 months. Most children presented with watery diarrhea of a limited duration. Three patients developed bloody diarrhea. One premature baby had diarrhea on day 40 in hospital and one 5-month-old boy with underlying type Ia glycogen storage disease developed diarrhea on day 21 in hospital. The nosocomial infection rate was 16.7% in this population. Some centers have reported nosocomial infection rates as high as 50%.[36] The majority of our patients recovered without specific treatment. Two exceptions included one patient who

received antibiotics for possible salmonellosis, and the other was a patient with acute myeloid leukemia who had neutropenia and fever. The positive EIA and RT-PCR results in a 14-month-old boy with chronic diarrhea indicated that astrovirus infection was a possible etiology. Although other pathogens for diarrhea were not routinely checked, our study did not show evidence of mixed infections. Our cases appeared in the autumn and winter months with a peak in December (5/12). Studies in temperate areas have shown astrovirus infections with a peak in the winter months.[19] It is interesting to note the higher prevalence of astrovirus-induced gastroenteritis among pediatric patients. This, in part, is a consequence of the relative immaturity of the pediatric immune system. Specifically, the secretory IgA that targets the astroviral antigens may be an integral component of the host defense system in older individuals. Human IgA levels are minimal during early life and do not reach adult levels until approximately 13 years of age.[37] In addition, B-cell isotype switching requires more time to occur than the duration of acute astroviral gastroenteritis.[3,37] Specific isotype-switching is correlated with CD4-positive T cell function.[37] This role for CD4-positive T lymphocytes has been suggested to be a factor in cases of persistent astroviral infections.[38] In the neonate, particularly a premature neonate, or those with primary or secondary causes of immunodeficiency, T and B lymphocyte functions are impaired.[39] This immunologic impairment is correlated with a higher susceptibility to viral and bacterial infections. This may have manifested in our study by the clinical status of Cases 1, 7 and 12, which were characterized by chronic diarrhea, acute myeloid leukemia with neutropenia, and prematurity, respectively.

A previous study has demonstrated two peaks in the serum presence of astrovirus (types 1 and 3) neutralizing antibodies.[24] The initial peak was prior to 6 months of age, which reflected transplacentally-acquired maternal (IgG1 and IgG3) neutralizing antibodies, the titers of which rapidly decreased to minimal levels after 6 months of age.[39] The second peak was the development of neutralizing antibodies by 6–9 years of age. These factors may apply to the susceptibility of Case 12, a premature neonate, who may not have had transplacentally-acquired maternal neutralizing antibodies. The transplacental acquisition of passive immunity only takes place during the third trimester, thus premature infants may acquire only minimal IgG1 and IgG3 anti-astrovirus antibody activities.[39]

Another interesting outcome of this study was the documentation of respiratory symptoms in approximately 50% of the reported patients. This finding may be a sign of a systemic infection rather than an isolated gastrointestinal infection in a subset of patients with astroviral infections. Similar clinical findings, specifically cough and rhinorrhea, were evident in patients with acute astroviral infections in pediatric patients in Japan.[40] Further work is required to elucidate this possibility.

Study limitations were that the patient population evaluated was limited in size and somewhat skewed as it represented only hospitalized patients and those evaluated in the emergency department.[30] Another limitation was the span of the study, which encompassed only a 1-year period. Even with these limitations, however, several findings are worthy of comment. Our results demonstrate that astrovirus may be an important source of gastroenteritis among children in Taipei. Furthermore, astroviral infections may be a cause of chronic diarrhea (1/12 patients) and may be nosocomially-acquired (2/12 patients), particularly among premature infants and those with immunodeficiency states. Our study results provide further information about the prevalence, serotypes and span of clinical spectra associated with astroviral infections in Taipei. Future studies should further elucidate the spectra of clinical presentations, variances in prevalence, and serotype distribution.

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